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Pseudocowpox virus Encodes a Homolog of Vascular Endothelial Growth Factor

Norihito Ueda,*[,1] Lyn M. Wise,*† Steven A. Stacker,† Stephen B. Fleming,* and Andrew A. Mercer*

*Virus Research Unit, Department of Microbiology, University of Otago, Dunedin, New Zealand; and †Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Melbourne, Victoria 3050, Australia

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We have identified a gene encoding a homolog of vascular endothelial growth factor (VEGF) in the *Pseudocowpox virus* (PCPV) genome. The predicted protein shows 27% amino acid identity to human VEGF-A. It also shows 41 and 61% amino acid identity to VEGFs encoded by orf virus (ORFV) strains NZ2 and NZ7, respectively. Assays of the expressed VEGF-like protein of PCPV ($PCPV_{VR634}$VEGF) demonstrated that $PCPV_{VR634}$VEGF is mitogenic for endothelial cells and is capable of inducing vascular permeability. $PCPV_{VR634}$VEGF bound VEGF receptor-2 (VEGFR-2) but did not bind VEGFR-1 or VEGFR-3. These results indicate that $PCPV_{VR634}$VEGF is a biologically active member of the VEGF family which shares with the ORFV-encoded VEGFs a receptor binding profile that differs from those of all cellular members of the VEGF family. It seems likely that the biological activities of $PCPV_{VR634}$VEGF contribute to the proliferative and highly vascularized nature of PCPV lesions.

Key Words: Parapoxvirus; Pseudocowpox virus; viral proteins; vascular endothelial growth factor (VEGF); VEGF-like protein; VEGF-E; vascular permeability factor; receptors, growth factor; receptor protein–tyrosine kinases; vascular endothelial cell growth factor receptor (VEGFR).

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a mitogen with specificity for endothelial cells and has the ability to induce vascular permeability (Ferrara and Davis-Smyth, 1997; Senger *et al.,* 1983). VEGF plays an important role in vasculogenesis and angiogenesis during embryonic development and in angiogenesis associated with a number of pathological conditions including tumor formation (Breier, 2000; Risau, 1997). The mammalian VEGF family consists of five members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PIGF). All are secreted, homodimeric glycoproteins that share 30 to 45% amino acid identity (Stacker and Achen, 1999). They exert their biological activities through a family of receptor tyrosine kinases, VEGF receptor 1 (VEGFR-1) (Flt-1), VEGFR-2 (Flk-1 or KDR), and VEGFR-3 (Flt-4) (Clauss, 2000). VEGF-A binds and activates both VEGFR-1 and VEGFR-2 but not VEGFR-3, while PIGF and VEGF-B bind only VEGFR-1. VEGF-C and VEGF-D bind both VEGFR-2 and VEGFR-3 but not VEGFR-1. The role of each receptor–ligand interaction has yet to be defined precisely but in general terms it appears that VEGFR-1 plays a role in vascular endothelial differentiation and migration, VEGFR-2 is the primary regulator of VEGF induced mitogenesis of endothelial cells, and VEGFR-3 is involved in lymphangiogenesis (Robinson and Stringer, 2001; Stacker *et al.,* 2002). In addition to shared amino acid sequences and functional characteristics, the VEGF family members are also structurally similar. All members of the VEGF family share a central domain referred to as the VEGF homology domain (VHD), which contains a cystine knot motif. (Achen *et al.,* 1998; Joukov *et al.,* 1997; Muller *et al.,* 1997b).

Pseudocowpox virus (PCPV) (synonym, paravaccinia virus, milker's nodule virus) is a member of the genus *Parapoxvirus,* the type species of which is *Orf virus* (ORFV) (Mercer *et al.,* 1997; Robinson and Lyttle, 1992). PCPV is maintained in cattle, while ORFV is maintained in sheep and goats, but both viruses infect humans. The clinical and histopathological features of PCPV lesions are indistinguishable from those of ORFV lesions. They both cause a contagious pustular dermatitis characterized by a progressive vesicle–pustule–scab formation in their clinical course in animals and humans. There are also some reports of extreme cases of human infection by parapoxviruses in which giant tumor-like lesions are described (Hunskaar, 1986; Pether *et al.,* 1986; Rogers *et al.,* 1989; Savage and Black, 1972). The histological features of PCPV and ORFV lesions include the endophytic strand-like epidermal downgrowths (rete ridges), papillary dermal edema, and marked capillary dilation and proliferation (Groves *et al.,* 1991).

ORFV has been shown to encode a new member of

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[1]To whom reprint requests should be addressed at Virus Research Unit, Department of Microbiology, University of Otago, P.O. Box 56, Dunedin, New Zealand. E-mail: norihito.ueda@stonebow.otago.ac.nz. Fax: +64-3-479-7744.

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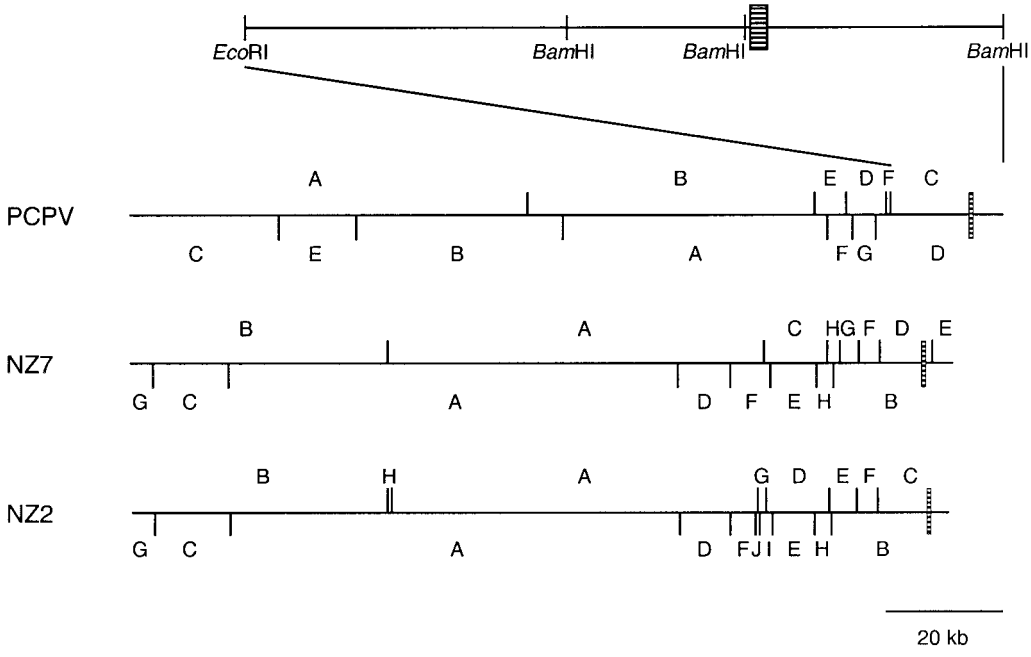

FIG. 1. Restriction enzyme cleavage maps of genomic DNA of PCPV strain VR634 and ORFV strains NZ2 and NZ7. Cleavage sites for *Eco*RI are marked by vertical bars extending above the map and *Hin*dIII sites by bars extending below. An 18-kb fragment (*Eco*RI C) is expanded and *Bam*HI sites are shown. Shaded bars indicate the locations of the VEGF-like genes.

the VEGF family and this provided a probable explanation for the proliferative and highly vascularized nature of ORFV-infected lesions (Lyttle *et al.,* 1994). ORFV is the only virus which has been reported to encode such a growth factor. It was noted that the predicted amino acid sequences of VEGF-like proteins of ORFV strains, NZ2 and NZ7 ($ORFV_{NZ2}$VEGF and $ORFV_{NZ7}$VEGF, respectively), are surprisingly different from each other, sharing only 45% identity (Lyttle *et al.,* 1994). This is despite the fact that the DNA sequences of other genes found in both isolates are almost identical and the VEGF-like genes occupy the same genomic location in both isolates. We and others recently showed that the ORFV VEGFs share some of the functional features of mammalian VEGF (Meyer *et al.,* 1999; Ogawa *et al.,* 1998; Wise *et al.,* 1999). Purified viral VEGFs were shown to stimulate the proliferation of vascular endothelial cells, to promote vascular permeability, and to bind VEGFR-2 and neuropilin-1, but not VEGFR-1 or VEGFR-3. This receptor binding profile is unique among the mammalian VEGF family, suggesting that the viral VEGFs constitute a new group within the family. We have constructed a recombinant ORFV in which the VEGF-like gene was disrupted and used this to demonstrate $ORFV_{NZ2}$VEGF function *in vivo.* During infection of sheep, the absence of an intact viral VEGF gene was associated with lesions that lacked the striking dermal vascularization and marked epidermal proliferation seen in wild-type virus infections (Savory *et al.,* 2000).

Numerous poxviruses have been shown to have genes which encode factors that determine virulence, pathogenesis, and host range (Alcami *et al.,* 1998; Spriggs, 1996). Such genes, including the ORFV VEGF genes, are frequently located in the termini of the genome. The similar histological features of PCPV and ORFV lesions suggested that PCPV might also encode a VEGF-like molecule and we hypothesized that such a gene might differ substantially in DNA sequence from the ORFV VEGFs, but occur in a similar genomic location. In this study, we identified a gene encoding a homolog of VEGF in the right terminus of the PCPV genome and conducted functional analysis of the expressed and purified protein.

RESULTS

Identification of a VEGF-like gene in PCPV

The published map of *Eco*RI digestion sites of PCPV strain VR634 includes an 18-kb fragment (*Eco*RI C) at the right genome terminus (Gassmann *et al.,* 1985; Robinson *et al.,* 1987). This *Eco*RI fragment occupies a genomic location similar to that of the VEGF gene of ORFVs (Fig. 1). Within PCPV *Eco*RI C we mapped three *Bam*HI sites (expanded in Fig. 1) and analysis of the DNA sequences adjacent to one of these *Bam*HI sites revealed evidence of a VEGF-like gene. The DNA sequence of this gene and the flanking regions was determined and is shown in Fig. 2, where it is aligned with the related sequences of ORFV strains NZ2 and NZ7.

Analysis of this region of PCPV DNA revealed that, in common with the ORFV sequences, it contains the partial sequence of a homolog of the vaccinia virus F9L gene,

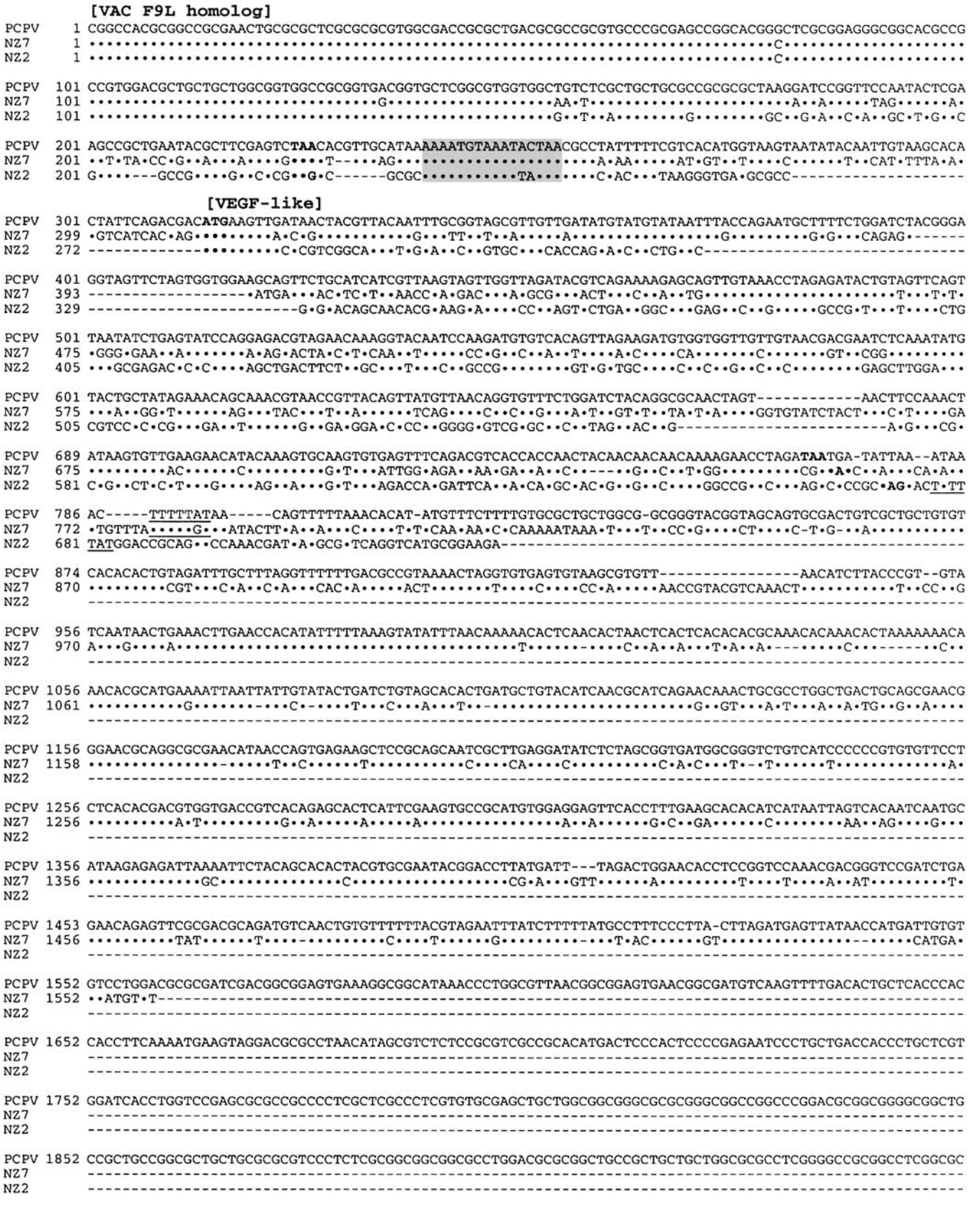

FIG. 2. Alignments of DNA sequences of the VEGF-like genes and flanking regions of PCPV and ORFV strains NZ7 and NZ2. DNA alignments of the VEGF-like genes were determined based on deduced amino acid sequences as aligned in Fig. 3. Nucleotides identical to the PCPV sequence are indicated by dots. Start codons and stop codons are shown in bold letters. Putative early promoter sequences are shaded and early transcriptional termination motifs are underlined. The ITR junctions of NZ7 and NZ2 are indicated.

FIG. 2—*Continued*

the complete sequence of a VEGF-like gene, and the partial sequence of a homolog of the ORF3 gene of NZ2 (Fleming *et al.,* 1991; Fraser *et al.,* 1990). The DNA sequence of the F9L-like gene and the ORF3-like gene of PCPV are very similar to ORFV sequences except within the last 40 bp of the F9L-like gene.

The noncoding region following the stop codon of the F9L-like gene is A + T-rich in all three parapoxviruses but the sequences are not well conserved except for an early promoter-like sequence (shaded box in Fig. 2) for the VEGF gene. This early promoter-like sequence of PCPV is identical with that of NZ7, while NZ2 has 2-bp mismatches with the others. An early transcriptional termination sequence (TTTTTNT, underlined in Fig. 2) (Fleming *et al.,* 1991) follows the stop codon of the VEGF gene in all three viruses. Early transcriptional control motifs identified in *Vaccinia virus* (Cochran *et al.,* 1985; Weir and Moss, 1987) are conserved in ORFV (Fleming *et al.,* 1991) and also appear to be a feature of PCPV.

TABLE 1

VEGF Sequence Comparisons

	PCPV	NZ7	NZ2	VEGF-A
PCPV	—	60.8	41.4	27.2
NZ7	65.1	—	45.1	24.5
NZ2	47.3	47.5	—	35.3
VEGF-A	38.5	34.9	46.0	—

Note. The percentages of amino acid sequence identities (above the diagonal) were calculated from the full length of the molecules as aligned in Fig. 3. Percentages of nucleotide sequence identities (below the diagonal) were calculated from DNA alignments based on the amino acid alignments shown in Fig. 3. VEGF-A is human isoform 121 (GenBank Accession Nos. AF214570 and AAF19659 for DNA and amino acid sequences, respectively).

The VEGF gene sequences of the three viruses share only 47 to 65% nucleotide sequence identity (Table 1). The PCPV VEGF gene has a G + C content of only 39% despite the high G + C content of the whole genome (63%, Wittek *et al.,* 1979). This feature is also seen in the NZ7 VEGF gene but is less apparent in the NZ2 VEGF gene. Alignment of the viral VEGF genes with human VEGF-A (isoform 121) nucleotide sequence revealed only a low level of sequence identity (Table 1). The sequences between the stop codon of the VEGF gene and the start codon of the ORF3 (-like) gene in PCPV, NZ7, and NZ2 share a relatively A + T-rich content but differ in length (Fig. 2).

VEGF amino acid sequence comparisons

The coding region of the PCPV VEGF is 4 to 19 codons longer than those of the ORFV VEGFs, largely as a result of a serine-rich domain in the N-terminus of the mature peptide. In common with the ORFV VEGFs, the VEGF encoded by PCPV strain VR634 ($PCPV_{VR634}$VEGF) includes a potential signal sequence as predicted by SignalP V2.0 (Nielsen *et al.,* 1997), an N-linked glycosylation site (Asn103–Thr105) and a threonine/proline-rich C-terminus that contains potential O-linked glycosylation sites (Thr140–Ser141, Thr144–Thr148) as predicted by NetOGlyc 2.0 (Hansen *et al.,* 1997). This program predicts further *O*-linked glycosylation sites that were not seen in $ORFV_{NZ2}$VEGF (Fig. 3).

Members of the VEGF family share a VHD containing eight cysteine residues that form a cystine knot motif and link the subunits of the antiparallel homodimer. All eight cysteines are conserved in $PCPV_{VR634}$VEGF and in the ORFV VEGFs. The predicted amino acid sequence of PCPV VEGF was compared with the 121-amino-acid isoform human VEGF-A and the VHD of the other members

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                        ___________________________ O   OO                    *                    *
PCPV          MKLITTLQFAVALLICMYNLPECFSGSTGGSSSGGSSSASSLSSWLDT-SEKSSCKPRDTVVQLISEYPGDVEQRYNPRC  79
NZ7           •••TA•••VV•••••••••••••V•Q•---------ND•PP•TND•MR•-LD••G••••••••Y•GE•••ESTNLQ•••••  71
NZ2           •••LVGILV••C•HQYLL•-------------------AD•NTKG•SEV-LKG•E••••PI••PVSETH•ELTS••F••P•  61
VEGF-A        •NFLLSWVHWS-•ALLL•LHHAKW•QAAPMAE-••GQNHHEVVKFM•V-YQR•Y•H•IE•L•DIFQ•••DEI•YIFK•S•  77
VEGF-B (VHD)                                                KVV••I•V-YTRAT•Q••EV••P•TV•LM•T•AKQLV•S•  72
VEGF-C (VHD)                                                EILKSI•NEWR•TQ•M••EVCIDVGK•FGVATNTFFK•P•  156
VEGF-D (VHD)                                                ETLKVI•EEWQRTQ•S••E•C•EVA••LGKSTNTFFK•P•  136
PlGF (VHD)                                                  EVVPFQEV-WGR•Y•RALERL•DVV••••SE••HMFS•S•  77

                      *  **#     *       NNN        O O           O        * *   OO  OOOOO
PCPV          VTVRRCGGCCNDESQICTAIETANVTVTVMLTGVSGSTGATS----NFQTISVEEHTKCKCEFQTSPPTTTTTKEPR     152
NZ7           •••K••S••••GDG•••••V••R•T••••SV••••S•S•TN•GVST•L•R•••T•••••D•IGR•TTTP-•••R•••R    148
NZ2           ••LM••••••••••LE•VPT•EV•••MELLGASG•••N•--------M•RL•FV••K••D•RPRFTTTPP•••RP••RRR 133
VEGF-A        •PLM•••••••••GLE•VPT•ES•I•MQI•RIKPHQGQH--------IGEM•FLQ•N••E•RPKKDRARQEKCDK••R    147
VEGF-B (VHD)  •••Q••••••P•DGLE•VPTGQHQ•RMQILMIRYPS•Q---------LGEM•L•••SQ•E•RPK                  127
VEGF-C (VHD)  •S•Y•••••••S•GLQ•MNTS•SYLSK•LFEIT•PL•Q•PK------PV•••FAN••S•R•MSK                  214
VEGF-D (VHD)  •N•F•••••••E••L••MNTS•SYISKQLFEIS•PLTSVPE------LVPVK•AN••G•••LPT                  194
PlGF (VHD)    •SLL••T•••G••NLH•VPV••••••MQLLKIRSGDRPS--------YVELTFSQ•VR•E•RPL                  133
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FIG. 3. Comparison of viral VEGFs with other members of the VEGF family. The deduced amino acid sequences of viral VEGFs were aligned with human VEGF-A (isoform 121) (GenBank Accession No. AAF19659) and the VHD of human VEGF-B (AAC50721), human VEGF-C (P49767), human VEGF-D (O43915), and human PlGF (CAA38698). Residues showing identity to $PCPV_{VR634}$VEGF are indicated by dots. Conserved cysteine residues of the cystine knot motif are indicated with asterisks. The predicted signal sequence of $PCPV_{VR634}$VEGF is indicated by a line above the sequence. The potential sites of N- and O-linked glycosylation of $PCPV_{VR634}$VEGF are indicated by italicized *N* and *O,* respectively. Asn88 of VEGF-A (indicated by a "#" symbol above the aligned sequences) is an accessible residue on the receptor binding face of VEGF-A (see Discussion).

of human VEGF family as well as ORFV VEGFs (Fig. 3 and Table 1). The PCPV VEGF gene encodes a predicted peptide with 61 and 41% sequence identity to $ORFV_{NZ7}$VEGF and $ORFV_{NZ2}$VEGF, respectively. The amino acid sequence identity of $PCPV_{VR634}$VEGF to VEGF-A (27%) is similar to that of the ORFV VEGFs. If the comparison is limited to the VHDs of viral and human VEGFs, then $PCPV_{VR634}$VEGF has 62, 44, 34, 35, 32, 32, and 31% sequence identities to $ORFV_{NZ7}$VEGF, $ORFV_{NZ2}$VEGF, VEGF-A, -B, -C, and -D, and PlGF, respectively.

Expression and purification of $PCPV_{VR634}$VEGF

To determine the function of $PCPV_{VR634}$VEGF, the coding region of the gene was cloned into a eukaryotic expression vector such that the expressed product would include the FLAG octapeptide at the C-terminus. The fusion protein was expressed in 293-EBNA cells and was purified by affinity chromatography with anti-FLAG M2 resin followed by elution with FLAG peptide. Analysis of this material by SDS–PAGE and silver staining (Fig. 4A) or Western blotting (Fig. 4B) demonstrated under nonreducing conditions a band of ≈48–52 kDa, whereas under reducing conditions a faster migrating band of ≈28–30 kDa was seen. The bands detected are consistent with $PCPV_{VR634}$VEGF being a disulfide-linked homodimer with a monomeric molecular mass of ≈29 kDa. This is larger than the predicted size of 15.0 kDa and is likely to be the result of N- and O-linked glycosylation at sites described above. To examine the glycosyl-

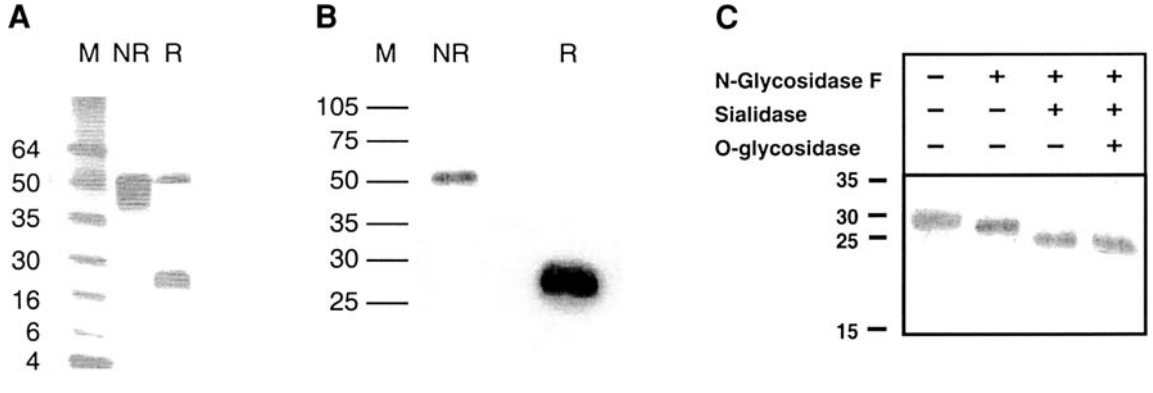

FIG. 4. Analysis of purified $PCPV_{VR634}$VEGF. The purified protein was resolved by SDS–PAGE and identified by (A) silver staining and (B) Western blotting with anti-FLAG monoclonal antibody. Molecular weight markers are indicated. R and NR show samples under reduced and nonreduced conditions, respectively. (C) Enzymatic removal of the N- and O-linked sugars. Purified $PCPV_{VR634}$VEGF was reduced by boiling in the presence of 2-mercaptoethanol and was then treated with the indicated combination of enzymes. The proteins were resolved by SDS–PAGE and visualized by silver staining.

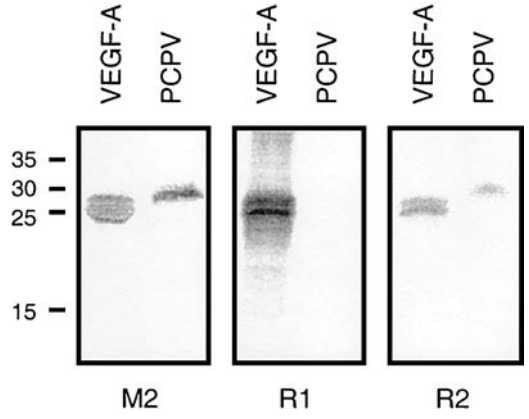

FIG. 5. The VEGFR binding specificity of $PCPV_{VR634}VEGF$. Soluble fusion proteins consisting of the extracellular domains of VEGFRs and the Fc portion of human IgG1 were used to assess the receptor binding specificity of $PCPV_{VR634}VEGF$. Biosynthetically labeled conditioned medium derived from 293-EBNA cells transfected with a plasmid encoding FLAG-tagged $PCPV_{VR634}VEGF$ or mouse VEGF-A (isoform 164) was mixed either with anti-FLAG M2-agarose (M2) or with VEGFR-1-Ig (R1) or VEGFR-2-Ig (R2) bound to protein A–Sepharose. Immunoprecipitated proteins were resolved by SDS–PAGE and visualized by PhosphoImager analysis.

ation state of $PCPV_{VR634}VEGF$, purified protein was deglycosylated by enzymatic removal of the N- and O-linked sugars. Following enzymatic treatment the proteins were resolved by SDS–PAGE and visualized by silver staining (Fig. 4C). Treatment with *N*-glycosidase F reduced the monomeric size of $PCPV_{VR634}VEGF$ by ≈2 kDa. The combined treatment of *N*-glycosidase F, sialidase, and *O*-glycosidase further reduced the monomeric size of $PCPV_{VR634}VEGF$ by ≈4 kDa. The monomeric mass of ≈23 kDa observed for deglycosylated $PCPV_{VR634}VEGF$ remains larger than the predicted size. This residual size difference may be due to incomplete enzymatic removal of the N- and O-linked sugars or the protein may not be resolved predictably by SDS–PAGE. A non-FLAG-tagged protein that comigrated with the nonreduced form of $PCPV_{VR634}VEGF$ was also detected by silver staining and was determined to be BSA derived from the fetal calf serum (FCS) used to supplement the culture medium (data not shown). Control preparations containing this protein but lacking $PCPV_{VR634}VEGF$ had no activity in the VEGF assays described below (data not shown; Savory *et al.,* 2000; Wise *et al.,* 1999).

$PCPV_{VR634}VEGF$ binds to soluble VEGFR-2 extracellular domains

To investigate the interactions between $PCPV_{VR634}VEGF$ and the VEGFRs, $PCPV_{VR634}VEGF$ was tested for its ability to bind to soluble Ig fusion proteins containing the extracellular domains of human VEGFR-1 or VEGFR-2. The Ig fusion proteins were expressed in 293-EBNA cells, precipitated from conditioned medium with protein A–Sepharose, and incubated with metabolically labeled FLAG-tagged $PCPV_{VR634}VEGF$ or FLAG-tagged mouse VEGF-A. Labeled proteins bound to the Ig fusion proteins were analyzed by SDS–PAGE and detected using a PhosphoImager (Fig. 5). Polypeptides corresponding to the size of $PCPV_{VR634}VEGF$ and mouse VEGF-A were precipitated by VEGFR-2-Ig. In contrast, VEGFR-1-Ig precipitated only VEGF-A. No labeled protein was precipitated from the medium of cells transfected with the expression vector lacking sequence encoding VEGF (data not shown). $PCPV_{VR634}VEGF$ was also shown to be unable to bind human VEGFR-3-Ig in a similar assay (data not shown). These data confirm that $PCPV_{VR634}VEGF$ can bind to VEGFR-2 but does not bind VEGFR-1 or VEGFR-3.

$PCPV_{VR634}VEGF$ binds and cross-links VEGFR-2

$PCPV_{VR634}VEGF$ was tested in a bioassay that detects ligands for VEGFR-2 (Fig. 6). The bioassay makes use of Ba/F3 cells in which activation of a chimeric receptor consisting of the extracellular domain of mouse VEGFR-2 and the transmembrane and cytoplasmic domains of the EpoR rescues cells from their dependence on IL-3, allowing the cells to proliferate in the absence of IL-3. VEGF-A, VEGF-C, VEGF-D, and $ORFV_{NZ2}VEGF$, which are ligands for VEGFR-2, all stimulate growth of this cell line in a specific and dose-dependent fashion (Achen *et al.,* 1998; Makinen *et al.,* 2001; Stacker *et al.,* 1999; Wise *et al.,* 1999). Purified $PCPV_{VR634}VEGF$ was able to induce detectable DNA synthesis in the bioassay cell line from a concentration of 25 ng/ml, whereas mouse VEGF-A was able to induce detectable DNA synthesis from a concentration of 5 ng/ml. Overall, $PCPV_{VR634}VEGF$ was about fourfold less potent in the

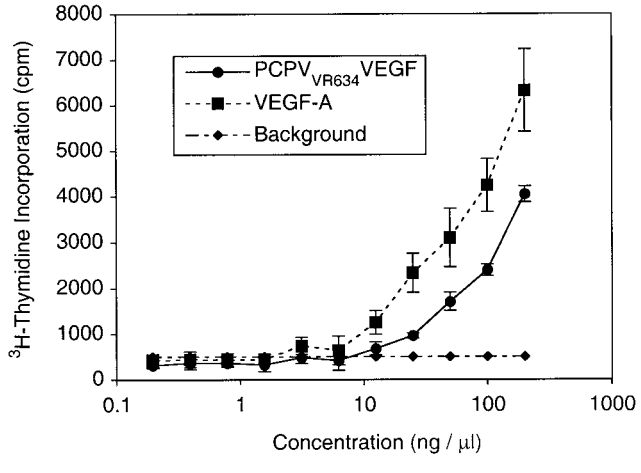

FIG. 6. $PCPV_{VR634}VEGF$ binds and cross-links VEGFR-2 in a VEGFR-2-specific bioassay. Purified proteins were tested for their ability to induce proliferation of Ba/F3 cells expressing a chimeric receptor consisting of the extracellular domain of mouse VEGFR-2 and the transmembrane and cytoplasmic domains of EpoR. Ba/F3 cells were washed with medium free of IL-3 and incubated in medium containing dilutions of $PCPV_{VR634}VEGF$, mouse VEGF-A, or control medium for 48 h at 37°C. DNA synthesis was quantitated by [^{3}H]thymidine incorporation and beta counting. Values are expressed as the mean ± SD of two replicate wells and are representative of four experiments.

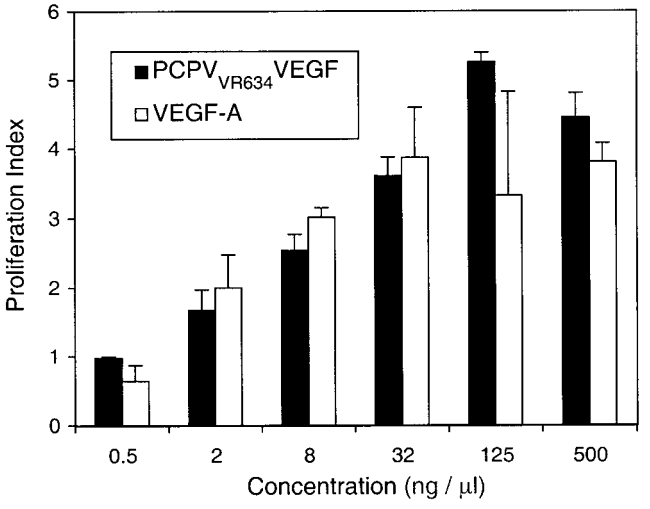

FIG. 7. Mitogenic activity of PCPV$_{VR634}$VEGF on HMVECs. HMVECs were cultured with dilutions of PCPV$_{VR634}$VEGF and mouse VEGF-A. Cell proliferation induced by purified proteins was quantitated by counting of cells after 72 h. Values are expressed as a proliferation index, which is the mean increase in cell number in four replicate wells divided by the mean background cell number in four wells which lacked purified protein. Error bars indicate 1 SD.

bioassay than VEGF-A. This reduced level of activity is similar to that reported for ORFV$_{NZ2}$VEGF, VEGF-C, and VEGF-D (Makinen *et al.,* 2001). These results clearly demonstrate that PCPV$_{VR634}$VEGF can bind to and cross-link the extracellular domains of VEGFR-2 and thereby has the potential to induce a proliferative response.

PCPV$_{VR634}$VEGF is mitogenic for endothelial cells

Members of the VEGF family of molecules show variable degrees of mitogenicity for endothelial cells. The mitogenic capacity of PCPV$_{VR634}$VEGF was tested using human microvascular endothelial cells (HMVECs). The cells were exposed to purified growth factors for 3 days before being assessed for growth. PCPV$_{VR634}$VEGF and mouse VEGF-A were able to stimulate an equivalent increase in the number of cells after 3 days compared with medium that did not contain added growth factors (Fig. 7). An analysis of variance (two-factor ANOVA with replication) did not reveal a statistically significant difference ($P > 0.05$) between the responses to PCPV$_{VR634}$VEGF and VEGF-A, indicating that PCPV$_{VR634}$VEGF was able to induce proliferation in HMVECs with a potency equivalent to that of VEGF-A.

PCPV$_{VR634}$VEGF induces vascular permeability

Members of the VEGF family of molecules also show varying abilities to induce vascular permeability. The ability of PCPV$_{VR634}$VEGF to induce vascular permeability was tested in the Miles assay. Guinea pigs were injected intradermally with various concentrations of the purified growth factors PCPV$_{VR634}$VEGF and mouse VEGF-A. In comparison to buffer alone, there was detectable and dose-dependent permeability induced by PCPV$_{VR634}$VEGF and VEGF-A (Fig. 8). PCPV$_{VR634}$VEGF, however, is considerably less potent as a vascular permeability factor than VEGF-A.

DISCUSSION

We determined the DNA sequence of 2.5 kb of the PCPV genome and within this region identified a VEGF-like gene. Purified PCPV$_{VR634}$VEGF was found to be a glycosylated disulfide-linked homodimer with a monomeric subunit of ≈29 kDa on SDS–PAGE. The VEGFR-2 bioassay using Ba/F3 cells expressing the extracellular ligand binding domain of VEGFR-2 revealed that PCPV$_{VR634}$VEGF is able to bind and cross-link VEGFR-2. As expected from these results, PCPV$_{VR634}$VEGF showed mitogenic activity on HMVECs. It also induced vascular permeability in the Miles assay. In addition we also found that PCPV$_{VR634}$VEGF, like VEGFs encoded by ORFV, did not bind VEGFR-1 or VEGFR-3 (Meyer *et al.,* 1999; Ogawa *et al.,* 1998; Wise *et al.,* 1999). The discovery that both PCPV and ORFV encode VEGF-like factors is consistent with the observed histopathological similarities in PCPV and ORFV lesions. We have recently demonstrated that ORFV$_{NZ2}$VEGF is responsible for a marked angiogenic response in ORFV-infected lesions by using a recombinant virus in which the VEGF-like gene was disrupted (Savory *et al.,* 2000). It therefore seems likely that the biological activities of PCPV$_{VR634}$VEGF also contribute to the proliferative and highly vascularized nature of PCPV lesions.

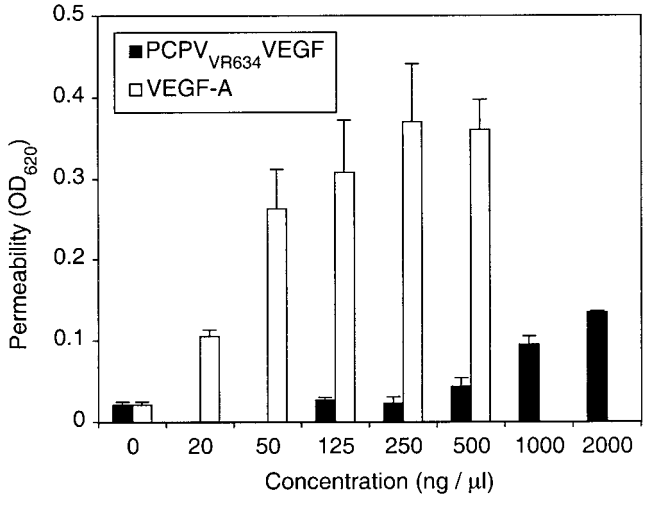

FIG. 8. Vascular permeability induced by PCPV$_{VR634}$VEGF in the Miles assay. Anesthetized guinea pigs were given intracardiac injections of Evans blue dye. Purified proteins were injected intradermally to shaved areas of the animals' backs. After 30 min, animals were sacrificed and the appropriate area of skin was excised. Samples were eluted in formamide and the absorbance reading at 620 nm was recorded. Values are expressed as the mean of two replicate injection sites and are representative of three experiments. Error bars indicate 1 SD of the mean.

Despite these functional similarities with the ORFV VEGFs, $PCPV_{VR634}$VEGF is also clearly divergent in sequence from both of the two major variants of VEGF encoded by strains of ORFV. The predicted amino acid sequence of $PCPV_{VR634}$VEGF has only 61 and 41% identity to those of $ORFV_{NZ7}$VEGF and $ORFV_{NZ2}$VEGF, respectively. These values are lower than generally reported between paired genes from the same genus of poxviruses (Mercer *et al.,* 2002).

The unusually high sequence variation between the PCPV VEGF and the ORFV VEGFs is mirrored in the ORFV VEGFs, with up to 31% amino acid sequence divergence among NZ2-like VEGFs (Mercer *et al.,* 2002) and even greater divergence between the NZ2 and NZ7 VEGFs. The source of this extensive sequence variation is unclear. A number of possible explanations can be considered, although in no case do they seem fully satisfactory and they must be considered speculative. One possible explanation of the variation between the viral VEGFs is that the major variants are derived from different members of the mammalian VEGF family, perhaps including members that have yet to be identified. Another possible explanation of the pronounced variation between the NZ2 and NZ7 VEGF sequences is raised by our analysis of the VEGF encoded by PCPV. The sequence relatedness between $PCPV_{VR634}$VEGF and $ORFV_{NZ7}$VEGF is greater than that between $ORFV_{NZ2}$VEGF and $ORFV_{NZ7}$VEGF (Table 1). This is true not only of the VEGF open reading frame but also of the immediately adjacent sequences (Fig. 2). It is only in sequences more distant from the VEGF genes that the expected close relationship between NZ2 and NZ7 become apparent (Fig. 2). This raises the possibility that strain NZ7 is a natural recombinant between strains of ORFV and PCPV and that the segment of DNA spanning the VEGF gene of strain NZ7 is derived from PCPV. The overlapping host ranges of parapoxviruses (Inoshima *et al.,* 2001; Robinson and Lyttle, 1992) could provide an opportunity for such recombination and a precedent for interspecies recombination among poxviruses is provided by *Malignant rabbit fibroma virus,* which is believed to have arisen by genetic recombination between *Shope fibroma virus* and *Myxoma virus* (Block *et al.,* 1985). However, this explanation too is not fully satisfactory in that the nucleotide sequence identity between the PCPV and NZ7 VEGF genes (65%) is substantially less than that between the most divergent pair of NZ2-like ORFV VEGF genes (76%) (Mercer *et al.,* 2002) and so demands even greater sequence divergence between the VEGF genes of PCPV isolates than is seen between ORFV isolates. Another species of parapoxvirus might be an alternative source of the NZ7 VEGF gene but VEGF genes have not been detected in other species, such as *Bovine papular stomatitis virus* (Büttner and Rziha, 2002).

Alignments of the viral VEGFs with full-length versions of members of the VEGF family reveal somewhat greater similarity to VEGF-A than to other groups of the family. However, the extent of sequence divergence seen in these alignments is not consistent with the viral VEGFs being a subgroup of the VEGF-A group. If the comparisons are restricted to the VHDs of representatives of the VEGF family groups, then $PCPV_{VR634}$VEGF shows a very narrow spread of amino acid sequence identity (31 to 35%), with little evidence that it is more closely related to any one of the family groups. These observations indicate that the viral VEGFs form a separate group within the VEGF family. This classification is supported by the phylogenetic analysis shown in Fig. 9, which illustrates the clear separation of the viral VEGFs from other groups of the VEGF family. The phylogenetic tree also illustrates both the extent of sequence divergence within the viral VEGF group and the closer relationship of $ORFV_{NZ7}$VEGF to $PCPV_{VR634}$VEGF than to $ORFV_{NZ2}$VEGF.

Studies of VEGF structure and function have identified

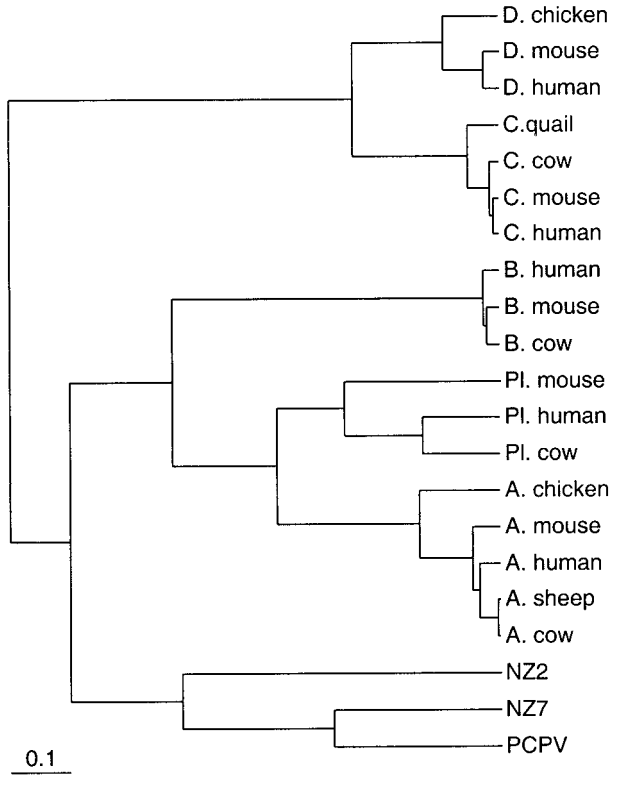

FIG. 9. Phylogenetic trees of viral VEGFs and other members of the VEGF family based on amino acid sequences of the VHD. Sequences used for the analysis are the viral VEGFs and the human VEGFs shown in Fig. 3 as well as VEGF-A of cow (AAA30502), sheep (CAA61677), mouse (AAB22254), and chicken (BAA24925), VEGF-B of cow (BAA77686) and mouse (AAC52823), VEGF-C of cow (BAA77687), mouse (P97953), and quail (CAA75799), VEGF-D of mouse (P97946) and chicken (AAM12733), and PlGF of cow (BAA77684) and mouse (CAA56453). The sequences were aligned manually and the genetic distance for each pair was estimated using the program EprotDist based on Dayhoff PAM matrix. Resulting distance matrix data were used to estimate phylogenies by the program Ekitsch by the Fitch–Margoliash method. Phylogenetic trees were visualized by TreeView-PPC Ver1.6.6 (Page, 1996) on Apple Macintosh.

receptor binding domains and have implicated specific residues in mediating binding to the VEGF receptors (Keyt *et al.,* 1996; Li *et al.,* 2000; Muller *et al.,* 1997; Wiesmann *et al.,* 1997). Our comparisons of ORFV VEGF variants and members of the mammalian VEGF family revealed only limited correlations between conserved residues and the ability to recognize specific VEGF receptors (Mercer *et al.,* 2002). A strong correlation is apparent between binding of VEGFR-2 and the presence of an Asn residue at a position equivalent to Asn88 of human VEGF-A (shown by a "#" symbol in Fig. 3). This residue is conserved in $PCPV_{VR634}$VEGF (Asn90, Fig. 3). Crystal structure determination of human VEGF-A has shown that Asn88 is an accessible residue on the VEGFR-2 binding face of VEGF-A (Muller *et al.,* 1997a).

Structural modeling of the ORFV VEGFs has allowed us to propose a possible basis for the inability of these VEGFs to bind VEGFR-1 (Mercer *et al.,* 2002). The structure determined by X-ray crystallography for the human VEGF-A homodimer revealed a groove at each end of the dimer that is postulated to play a role in its binding to VEGFR-1 (Wiesmann *et al.,* 1997). Our models of ORFV VEGFs suggest that this groove is blocked in the ORFV VEGFs and this may therefore contribute to their inability to bind VEGFR-1. Modeling of $PCPV_{VR634}$VEGF indicated that, consistent with its inability to bind VEGFR-1, it too is unlikely to have a groove able to assist in binding VEGFR-1 (data not shown). Structural determinations in conjunction with site-directed mutagenesis and receptor binding assays will be required to assess the validity of these models.

Our analysis of 2.5 kb of PCPV DNA sequence confirms the classification of PCPV as a member of the *Parapoxvirus* genus but as a species separate from ORFV. The PCPV sequence revealed evidence of three genes, all of which have significant sequence relatedness to genes of ORFV. Furthermore, these genes occur in the same order and orientation in both viruses, they occupy similar genomic locations, and two of the genes have not been reported in other poxviruses. These observations support the inclusion of PCPV and ORFV in the same genus, whereas a comparison of the PCPV sequence of a F9 homolog and the ORF-3-like gene with two ORFV isolates validates the separation of PCPV and ORFV as two species. Our data therefore support the conclusions of the only previous report of DNA sequence of PCPV (Inoshima *et al.,* 2001).

The identification of $PCPV_{VR634}$VEGF as another major subgroup within the viral VEGFs will provide opportunities to compare the structural and functional characteristics of these factors with their mammalian counterparts. Such comparisons are likely to be useful in identifying elements of these growth factors that mediate their interactions with the family of VEGF receptors and mediate their wide range of biological activities.

MATERIALS AND METHODS

Cells and viruses

Primary bovine testis (BT) cells were grown in Eagle's minimal essential medium containing 10% FCS and 5% lactalbumin hydrolysate. PCPV strain VR634 (Gassmann *et al.,* 1985) was propagated in BT cells as described previously (Robinson *et al.,* 1982).

Purification of virus and DNA extraction

Viral particles propagated in BT cells were purified in sodium diatrizoate gradients as described previously (Robinson *et al.,* 1982). Purified virions were treated with proteinase K and SDS followed by isolation of viral DNA in guanidine HCl–CsCl gradients as described previously (Mercer *et al.,* 1987).

DNA cloning and sequence analysis

The general methods used have been described previously (Fraser *et al.,* 1990). Double-stranded DNA templates were prepared and sequenced by procedures recommended by Applied Biosystems Inc. (ABI). Reagents used for sequencing were supplied by ABI, and the products of sequencing reactions were analyzed with an ABI Model 377 sequencing system. The nucleotide sequences of both strands of the region under study were determined and were assembled using the program SeqManII Ver. 5.00 (DNASTAR, Inc.). Nucleotide and predicted amino acid sequences were compared with database sequences using the BLAST suite of programs (http://www.ncbi.nlm.nih.gov/BLAST/).

Expression of the VEGF-like gene

The PCPV VEGF-like gene was expressed as a fusion protein using a derivative of the mammalian expression vector pEF-BOS (Mizushima and Nagata, 1990). A DNA fragment containing the VEGF-like gene was amplified by PCR using virus DNA as template with two primers: GF5-FLAG (5′-AGCGCCCGGCGCGCCAGAAGTTGATAACTACGT-3′, *Asc*I site underlined) and GF6-FLAG (5′-ACTCGAACGCGTTCTAGGTTCTTTTGTT-3′, *Mlu*I site underlined). The PCR product was digested with *Asc*I and *Mlu*I and inserted into the pEF-BOS-I-FLAG expression vector (kindly provided by Clare MacFarlane, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) at the *Asc*I site, immediately upstream of the DNA sequence encoding the FLAG octapeptide (IBI/Kodak), so that the FLAG octapeptide would be fused to the C-terminus of the synthesized VEGF-like peptide and this fusion protein would be secreted from transfected cells (Wise *et al.,* 1999). This plasmid was transfected into 293-EBNA cells using FuGENE6 (Roche) as recommended by the manufacturer. After 7 days of incubation, conditioned cell culture medium was collected and clarified by centrifugation before FLAG-tagged proteins were

recovered by affinity chromatography using anti-FLAG M2-agarose (Sigma). Alternatively, an expression cassette was excised from the above plasmid and inserted into the expression vector pAPEX-3 (Evans *et al.,* 1995) (kindly provided by Steven Squinto, Alexion Pharmaceuticals, New Haven, CT) to allow stable expression of the VEGF-FLAG fusion protein and the protein was purified from conditioned medium. Non-FLAG-tagged mouse VEGF-A (isoform 164) was expressed in CHO cells and purified (Stacker *et al.,* 1999). Mouse VEGF-A (isoform 164) was also expressed in the form of a fusion protein with the FLAG peptide using an pAPEX-3-derived vector as described above for $PCPV_{VR634}$VEGF. This protein was used in binding assays utilizing soluble VEGFR extracellular domains (see below).

SDS–PAGE and immunoblotting

Purified $PCPV_{VR634}$VEGF was boiled in the SDS sample buffer with or without 2-mercaptoethanol and resolved by SDS–PAGE (Laemmli, 1970). The protein was transferred to nitrocellulose membrane and HRP-conjugated anti-FLAG M2 monoclonal antibody (Sigma) was used to detect the FLAG-tagged protein (Achen *et al.,* 1998).

Deglycosylation of $PCPV_{VR634}$VEGF

Purified protein (0.6 μg) was diluted in 0.05 M sodium phosphate (pH 7) containing 0.1% SDS and 20 mM 2-mercaptoethanol and boiled for 5 min. The mixture was cooled on ice and Tween 20 was added to 0.75%. Five units of *N*-glycosidase F (Roche), 5 mU of sialidase (Neuraminidase, Roche), or 1.25 mU of *O*-glycosidase (Roche) was added in the following combinations: *N*-glycosidase F alone, *N*-glycosidase F and sialidase, or *N*-glycosidase F, sialidase, and *O*-glycosidase. Alternatively no enzyme was added. The mixtures were then incubated at 37°C for 3 h. The proteins were resolved by SDS–PAGE and visualized by silver staining.

Binding assays with soluble VEGFR extracellular domains

The details of these assays have been described elsewhere (Wise *et al.,* 1999). Briefly, 293-EBNA cells were transfected with plasmids encoding the soluble human receptor–Ig fusion proteins VEGFR-1-Ig or VEGFR-2-Ig. The soluble fusion proteins were precipitated from the conditioned medium using protein A–Sepharose beads. Beads were then combined with conditioned medium from 293-EBNA cells that had been transfected with expression plasmids encoding $PCPV_{VR634}$VEGF or mouse VEGF-A and biosynthetically labeled with [^{35}S]Cys/Met. Precipitated VEGFs were separated by SDS–PAGE and detected by using a PhosphoImager analyzer (Fujifilm).

Bioassay to monitor binding to the extracellular domain of VEGFR-2

The bioassay has been described previously (Wise *et al.,* 1999). Briefly, purified proteins were tested for their ability to induce proliferation of Ba/F3 cells that express a chimeric receptor consisting of the extracellular domain of mouse VEGFR-2 and the transmembrane and cytoplasmic domain of the mouse erythropoietin receptor (EpoR). DNA synthesis of the cells was quantitated by [^{3}H]thymidine uptake and beta counting.

Mitogenesis assay with endothelial cells

The purified proteins were tested for mitogenic activity on HMVECs. Cells grown in EGM-MV (Cambrex) containing 10% FCS and growth supplements, including epidermal growth factor and bovine brain extract as specified by the manufacturer, were removed with trypsin, washed, and aliquoted at 10^4 cells/well in a 24-well plate. Cells were allowed to adhere for 6–16 h at 37°C before test or control samples diluted in EGM-MV containing 2% FCS but lacking the additional supplements were added. After 72 h of growth at 37°C cell proliferation was quantitated by removing the cells with trypsin and counting cell numbers with a hemocytometer (Wise *et al.,* 1999).

Miles assay for vascular permeability

The Miles vascular permeability assay was performed using guinea pigs as described previously (Wise *et al.,* 1999). In brief, anesthetized animals were given an intracardiac injection of Evans blue dye. Purified proteins were injected intradermally to shaved areas of the animal's back. After 30 min the animal was sacrificed, the skin was excised, and samples were eluted in formamide. The amount of dye extracted from the lesion was determined by measuring OD 620 nm.

VEGF NOMENCLATURE

The ORFV-encoded VEGFs have been classified by some researchers as VEGF-E in recognition of the unique receptor binding profile of the viral factors as well as the amino acid sequence divergence they show from cellular VEGFs (Meyer *et al.,* 1999; Ogawa *et al.,* 1998). In light of the extensive variations in amino acid sequence seen between individual viral VEGFs, we believe that to refer to a particular viral VEGF as VEGF-E may be misleading. For example, the sequence identity between the VEGFs encoded by ORFV strains NZ2 and NZ7 is no greater than that between human VEGF-A and human VEGF-B. To name the PCPV-encoded VEGF and both of the ORFV-encoded VEGFs as VEGF-E would misrepresent the sequence variation between them and suggest a degree of shared function or origin that further investigations may not support. Such a nomenclature is likely to give rise to confusion as further comparative analyses

of these factors are published. We believe that it is preferable to restrict the use of a single uppercase letter suffix (VEGF-A, VEGF-B, etc.) to the classification of types of cellular VEGF. We have therefore used the recommended abbreviation of the virus name (van Regenmortel *et al.,* 2000) as a prefix and appended to it a strain designation in subscript. This allows a distinction to be made between the major variants of ORFV-encoded VEGF (NZ2 and NZ7). In accordance with this nomenclature, the vascular endothelial growth factor encoded by *Pseudocowpox virus* strain VR634 is designated $PCPV_{VR634}VEGF$.

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